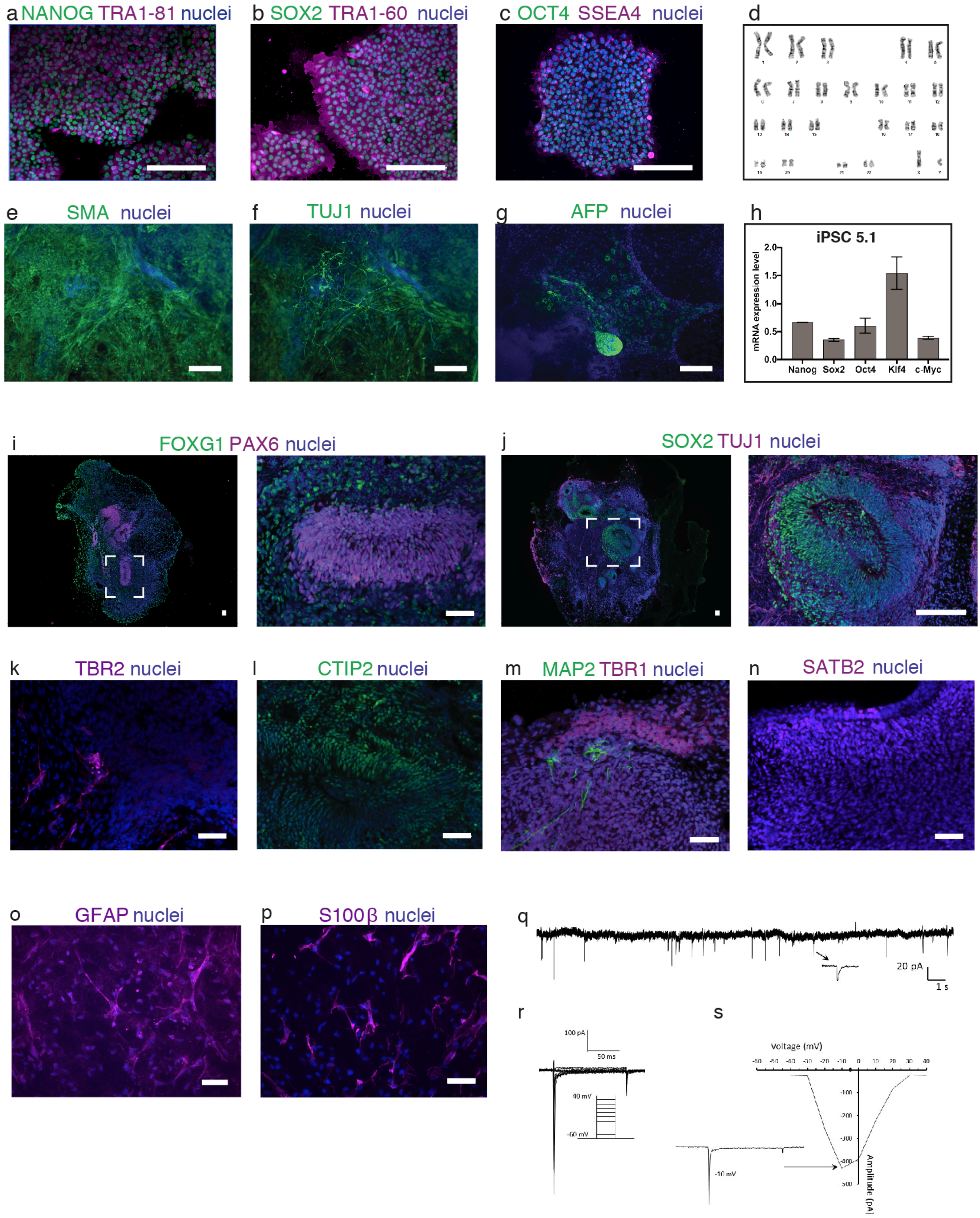
Supplementary Information "Microglia Innately develop within cerebral organoids"

Ormel et al.



Supplementary Figure 1. iPSC5 characterization and neuronal identity in organoids

a to c- Stem cell markers NANOG and TRA1-81 (a), SOX2 and TRA1-60 (b), and OCT4 and SSEA4 (c) expression in iPSC5 line. Scale bar 200 μm.

d- Karyogram of iPSC 5.

e to g- Pluripotency potential was evaluated by spontaneous differentiation assay followed by immunohistochemistry for smooth muscle actin (SMA, e), β-III Tubulin (TUJ1, f), and α-fetoprotein (AFP, g). Scale bar 200 μm.

h- mRNA expression of stem cell markers by qRT-PCR (normalized to *ACTB*) with a commercial embryonic stem cell line (HUES6). mRNA expression of iPSC 5 relative to HUES6.

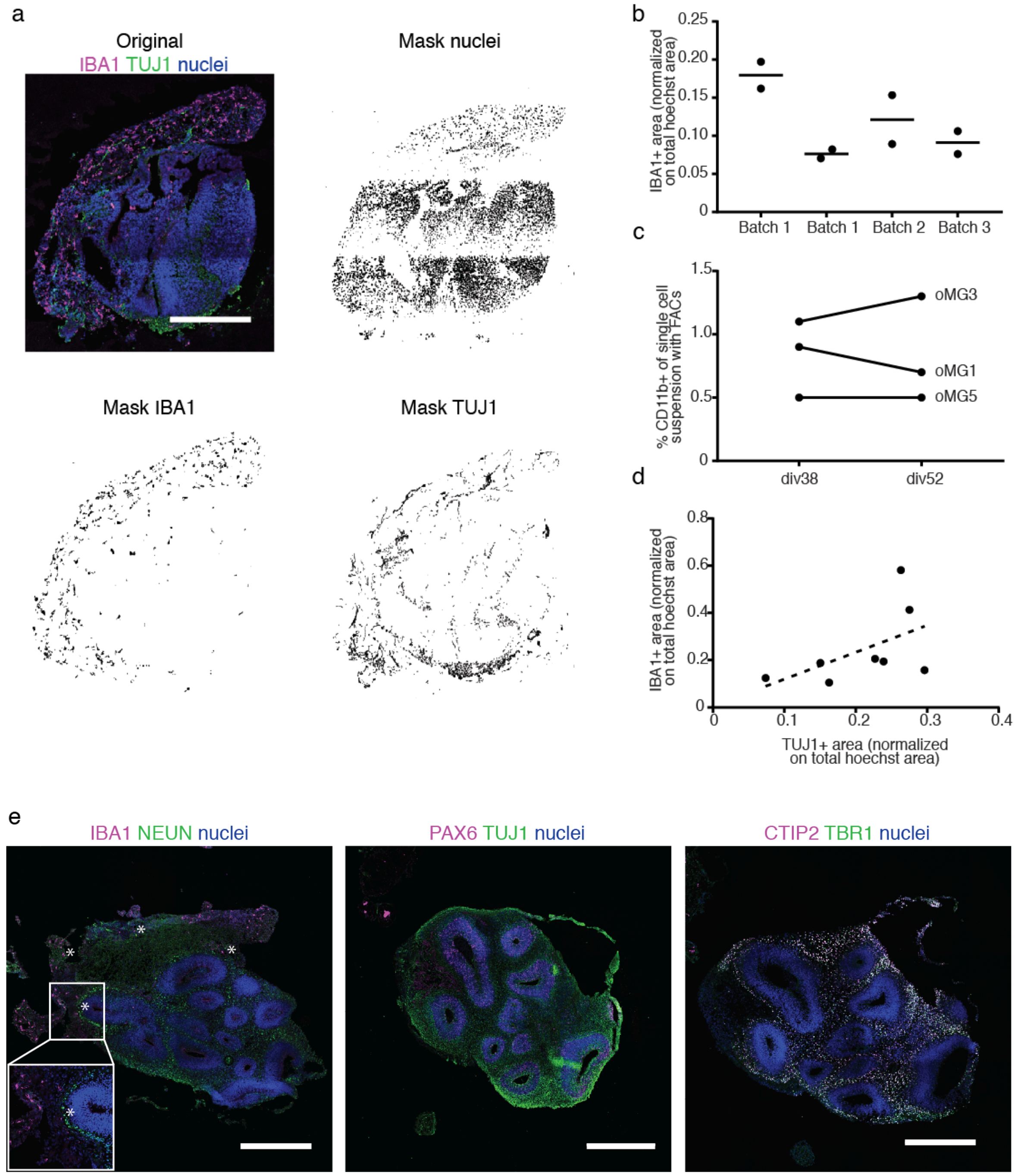
i- Dorsal forebrain identity of organoids assessed by FOXG1 and PAX6 immunostainings at day 31. Representative pictures of organoids from iPSC 1 are shown. (right panel is a close-up of the left panel) Scale bars 200μm.

j- Neuronal features of the organoids was evaluated by immunohistochemistry for progenitor marker SOX2 and pan-neuronal marker TUJ1 at day 31. Representative pictures of organoids derived from iPSC 1 are shown. (right panel is a close-up of the left panel) Scale bars 200 μm.

k, l and m- Presence of TBR2$^+$ progenitors (k), cortical deep layer neurons expressing CTIP2 (l) and TBR1 (m), and mature neurons MAP2 (m) in cerebral organoids at day 31. Representative pictures of organoids from iPSC 1 are shown. Scale bars 200 μm.

n, o and p- Superficial layers were assessed by SATB2 expression at day 31 (n) and presence of astrocytes evaluated by expression of GFAP (o) and S100β (p) at day 52. Representative pictures of cerebral organoids from iPSC 1 are shown. Scale bars 200 μm.

q, r, s- Glutamatergic spontaneous excitatory postsynaptic potentials (sEPSCs) detected at a holding potential of -65 mV. Voltage dependent sodium currents, necessary for the generations of spikes, could be elicited (q). Example of a single sodium current evoked at -10 mV is shown (r). IV plot depicting the typical course of the voltage dependency of the sodium currents is shown (s). Cerebral organoids from iPSC 1 were used for the electrophysiology.



Supplementary Figure 2. The quantity of microglia is similar between batches, donors, and timepoints in culture and co-mature with neurons

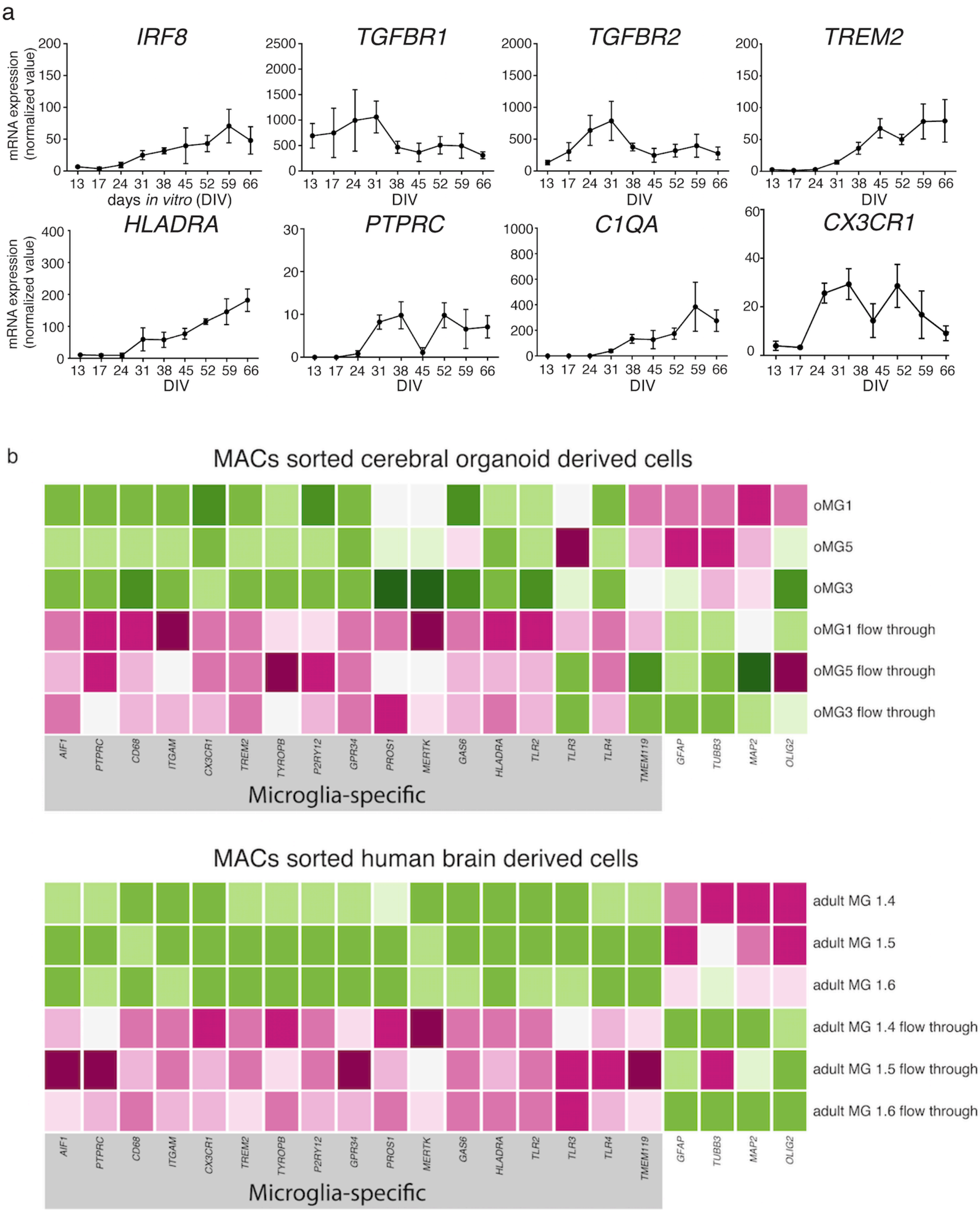
a- Mask images created by an automated macro in FIJI to quantify the fraction of nuclei, IBA-1, and TUJ1 positive area of a tiled image of a fluorescent staining. The fluorescent channels were split and a separate threshold was applied to enable further analyses. Representative pictures of cerebral organoids from iPSC 1 are shown. Scale bar 500 μm.

b- Quantification of IBA-1/nuclei ratio from tiled fluorescent images of 2 sections per organoid. The variation between batches is similar to the variation between organoids of the same batch ranging from 0.05-0.2.

c- Percentage of CD11b^{+} cells in organoid single cell suspension when sorted with flow cytometry. Organoids from three donors were used for this experiment (iPSC 1, 3, and 5) at two timepoints (38 and 52 days *in vitro*). The mean percentage of CD11b^{+}/CD45^{+}cells of donors iPSC 1, 3, and 5 was 0.83% ± 0.3 (SD) at both time points for oMG (n = 6)

d- The increase of IBA1^{+} is positively correlated with TUJ1. Each data-point reflects the IBA-1 and TUJ1 fraction, normalized to nuclei, of one tiled image of organoids from three separate batches.

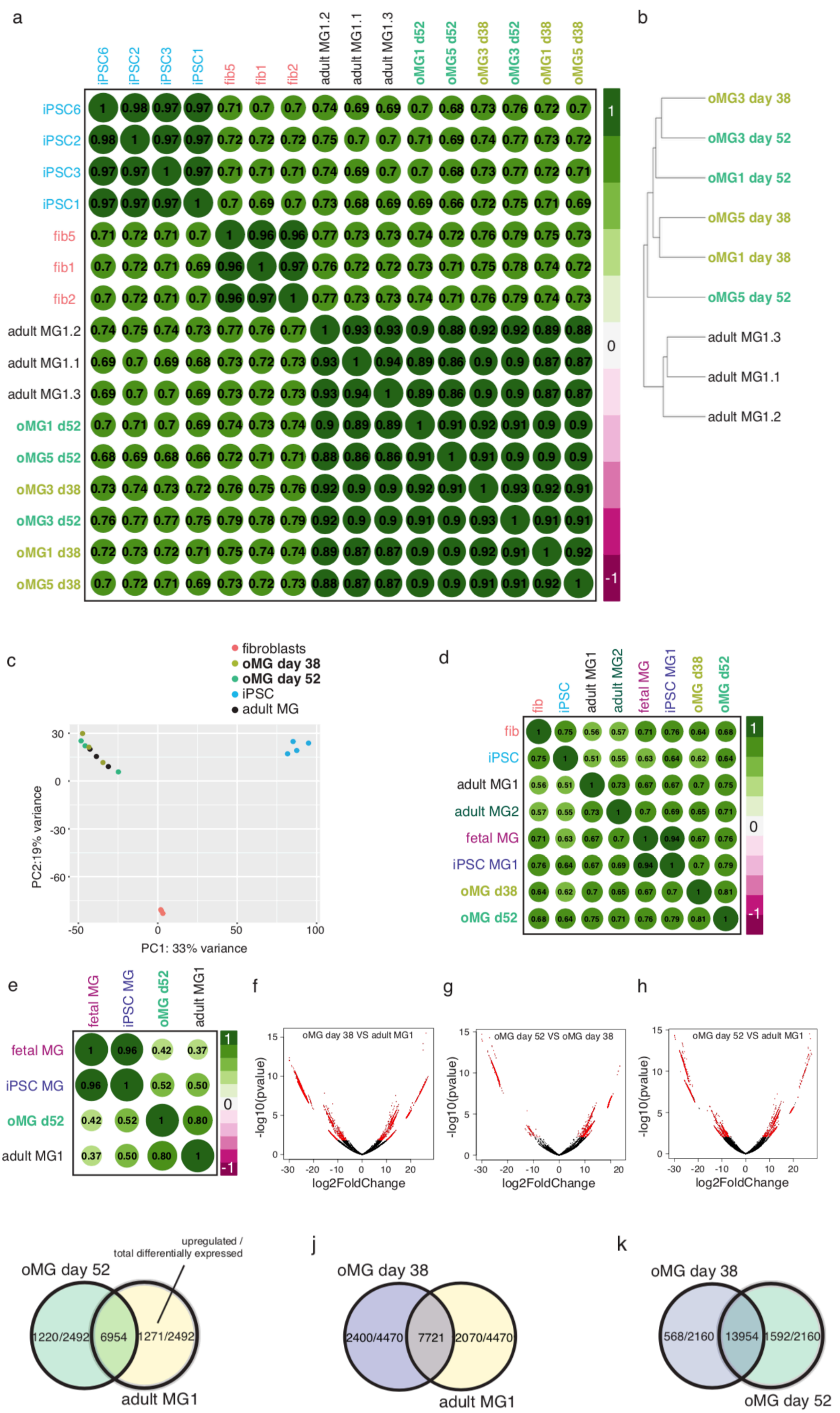
e- Neuronal identity and cyto-architecture is maintained in organoids containing microglia as shown in tiled pictures from sections of one organoid after 66 days in culture. Co-staining for: microglia (IBA-1) and mature neurons (NEUN) (* indicates NEUN^{+} cells in close proximity with IBA-1^{+} cells, left panel); radial glia (PAX6) and a pan-neuronal marker (TUJ1, middle panel); and for a deep cortical layer marker (CTIP2) and a post-mitotic projection neuron marker (TBR1, right panel). Scale bar 500 μm.



Supplementary Figure 3. oMG express microglia-specific genes that could be measured in the whole organoid but also when the microglia population is enriched

a- Graphs depicting mRNA expression levels of microglia-specific genes in organoids. mRNA levels were determined by qRT-PCR and normalized to the geomean of the reference genes *SDHA2* and *ACTB*. Data represent the mean of four batches consisting of two organoids per batch per time-point. All batches consisted of organoids derived from iPSC 1. Error bars represent the standard error of the mean (SEM).

b- Magnetic automated cell sorting validation in oMG (upper panel) and adult MG (lower panel) by qRT-PCR by using a panel of classical microglia genes but also genes that should not be expressed by microglia (*GFAP*, *TUBB3*, *MAP2*, and *OLIG2*). mRNA levels were compared with the flow through fraction (oMG flow through and adult MG flow through). Data was log transformed and scaled for each sample to visualize the expression pattern. mRNA levels were determined by qRT-PCR and normalized to the geomean of the reference genes *SDHA2* and *ACTB*. n = 3 separate experiments in which oMG were isolated with CD11b$^+$ magnetic cell sorting from 8 organoids per experiment and also an n = 3 separate experiments to enrich adult MG with CD11b$^+$ magnetic cell sorting from fresh human brain tissue. (*$p < 0.05$)


a
iPSC6
iPSC2
iPSC3
iPSC1
fib5
fib1
fib2
adult MG1.2
adult MG1.1
adult MG1.3
oMG1 d52
oMG5 d52
oMG3 d38
oMG3 d52
oMG1 d38
oMG5 d38
b
oMG3 day 38
oMG3 day 52
oMG1 day 52
oMG5 day 38
oMG1 day 38
oMG5 day 52
adult MG1.3
adult MG1.1
adult MG1.2
c
fibroblasts
oMG day 38
oMG day 52
iPSC
adult MG
PC1: 33% variance
PC2:19% variance
d
fib
iPSC
adult MG1
adult MG2
fetal MG
iPSC MG1
oMG d38
oMG d52
e
fetal MG
iPSC MG
oMG d52
adult MG1
f
oMG day 38 VS adult MG1
g
oMG day 52 VS oMG day 38
h
oMG day 52 VS adult MG1
-log10(pvalue)
log2FoldChange
i
upregulated /
total differentially expressed
oMG day 52
1220/2492
6954
1271/2492
adult MG1
j
oMG day 38
2400/4470
7721
2070/4470
adult MG1
k
oMG day 38
568/2160
13954
1592/2160
oMG day 52

Supplementary Figure 4. Correlation among microglia-like cells

a- Spearman correlation analysis between oMG day 38, oMG day 52, adult MG 1, iPSC and fibroblast samples. DESeq2 rlog transformed raw gene counts of all genes annotated after removal of common genes (FDR > 0.05, sum of raw read counts > 0) between the samples were used as input. Size and color of circles indicate the strength and direction of the correlation, respectively.

b- Unsupervised hierarchical cluster analysis on DESeq2 rlog transformed raw counts of oMG day 38, oMG day 52, and adult MG1 based on all genes after removal of common genes (FDR > 0.05, sum of raw read counts > 0) between samples.

c- Principal component analysis on DESeq2 rlog transformed raw counts of oMG day 38, 52, adult MG, iPSC and fibroblasts.

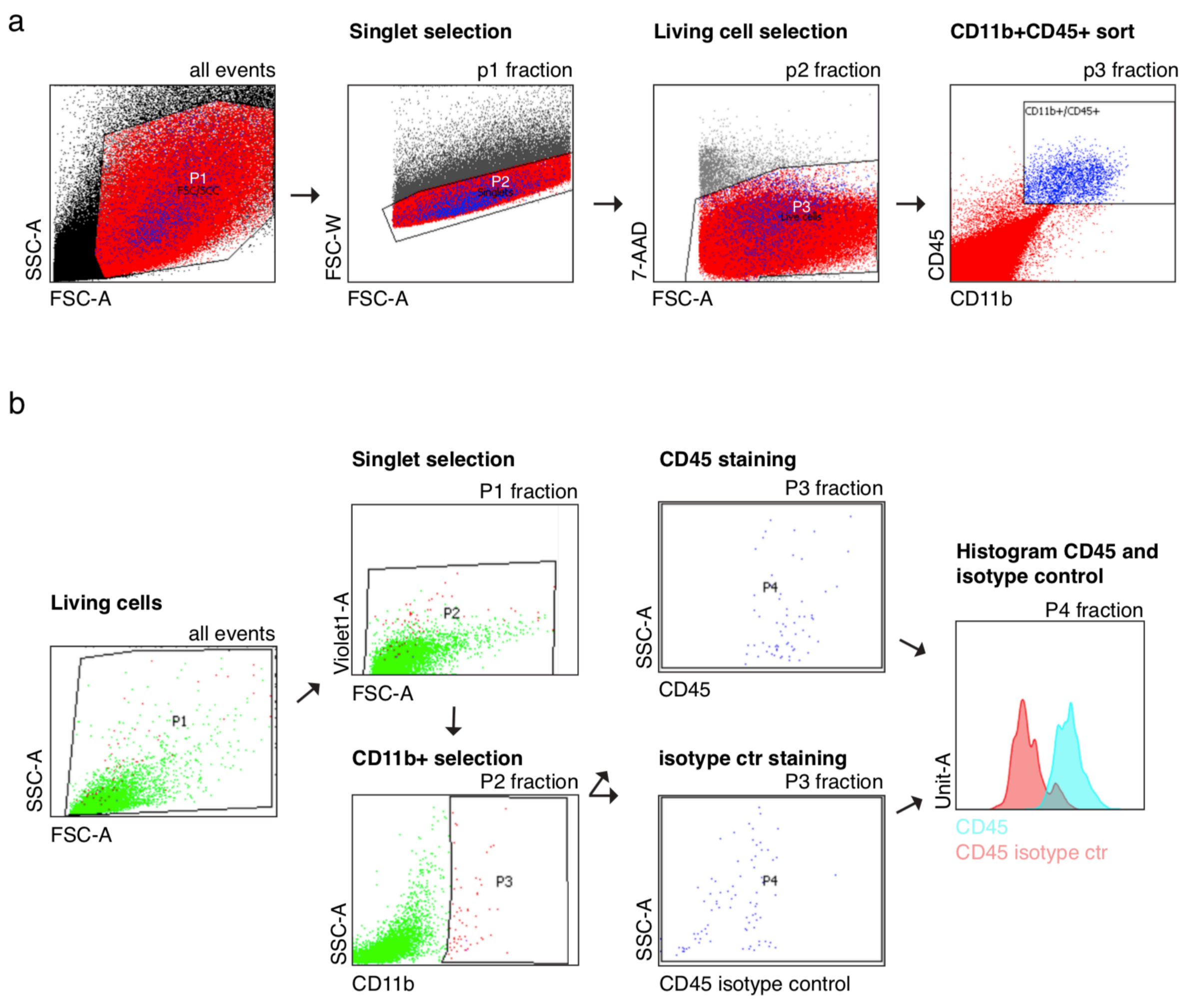
d- Spearman correlation analysis between oMG day 38, 52, adult MG1, fetal MG, iPSC MG and adult MG2 scaled log transformed FPKM values of genes used in figure 3f. Median log transformed FPKM values for biological replicates were used as input for the correlation analysis.

e- Spearman correlation analysis between oMG day 52, adult MG, fetal MG, and iPSC MG scaled log2 FPKM values of a panel of transcription factor families that recognize microglia-related motifs. Median log transformed FPKM values for biological replicates were used as input for the correlation analysis.

f, g, and h- Volcano plots show differentially expressed genes (FDR < 0.05 in red) between day 38 oMG vs adult MG1 (f), day 52 vs day 38 oMG (g), and day 52 oMG vs adult MG1 (h).

i, j, and k- Venn diagrams show common expressed (FDR > 0.5, sum of raw read counts > 0) and differentially expressed genes (FDR < 0.05, enriched genes in sample/total amount of differentially expressed genes) between day 52 oMG vs adult MG1 (i), day 38 oMG vs adult

MG1 (j), and day 38 vs day 52 oMG (k) after shrinkage correction of the log2Fold change and removal of identified common genes between iPSC, fibroblasts, oMG and adult MG1.



Supplementary Figure 5. Flow cytometry gating strategies used for cell sorting and protein expression quantification

a- Gating strategy to sort CD11b+CD45+ single living cells from the organoid/brain single cell suspensions. Representative gates of oMG enrichment of iPSC 3 are shown.

b- Gating strategy to quantify protein expression of CD11b+ cells gated from organoid/brain single cell suspension. Isotype controls of respective antibodies were used to accurately determine the protein expression. Representative gates of CD11b+ stained cells of iPSC 1 are shown.

Abbreviations: SSC-A = sideward scatter area; FSC-A = foreward scatter area; FSC-W = foreward scatter width.

Supplementary Table 1. Comparative overview of adaptations in the organoid differentiation protocol used in this study and the original protocol of Lancaster et al. 2014

Description		Ormel et al. 2018	Lancaster et al. 2014
Embryoid bodies generation	medium	hES4 (-P/S; +FBS); Y27 (1:100)	hES4 (-P/S; +FBS); Y27 (1:100)
	plates	AggreWell 800, (300 microwells/well)	V- bottom ULS 96 well plate
	Volume	2 mL (1.75x10^6 cells per mL)	150 µL (6x10^4 cells/mL)
	cells per embryoid body (EB)	Approx. 11500 cells	9000 cells
Germ layer differentiation I	timing	up to day 4	up to day 4
	medium	hESC4 ; Y27 (1:100)	hESC4 ; Y27 (1:100)
	plates	flat bottom ULA 96 well plate	V- bottom ULA 96 well plate
	EB size	320 (+/- 40) μm	> 350-400 μm
Germ layer differentiation II	timing	Days 4 to 6	Days 4 to 6
	medium	hES0	hES0
	plates	flat bottom ULA 96 well plate	V- bottom ULA 96 well plate
	EB size	330 (+/- 37) μm	350-600 μm
Induction of neural ectoderm	timing	days 6 to 12	days 6 to 9
	medium	NIM with 0.1 μg/mL Heparin	NIM with 1 μg/mL Heparin
	plates	flat bottom ULA 96 well plate	flat bottom ULA 24 well plate
	EB size	330-570 μm	500-600 μm
Transfer to Matrigel	timing	Day 13	Day 11
	medium	Differentiation medium without RA	Differentiation medium without RA
	plates	60 mm petri dish	60 mm tissue culture dish
Transfer bioreactor	timing	Day 17	Day 15
	medium	Differentiation medium with RA	Differentiation medium with RA
	platform	Spinning bioreactor	Spinning bioreactor
	speed	27.5 rpm	25 rpm

Supplementary Table 2. Gene panels consisting of transcription factor genes important for microglia functioning *in vivo*

Transcription family	Transcription factors important for microglia functioning *in vivo*
PU.1	*SPI1*
CTCF	*CTCF*
IRF	*IRF1*
	IRF2
	IRF3
	IRF8
	IRF9
RUNX	*RUNX1*
	RUNX2
AP-1	*JUN*
	JUNB
	JUND
	FOS
	FOSB
	FOSL2
	ATF4
	BATF
	BATF2
	BATF3
C/EBP	*CEBPA*
	CEBPB
	CEBPG
MEF2	*MEF2A*
	MEF2B
	MEF2C
	MEF2D
SMAD	*SMAD3*
MAF	*MAF*
	MAF1
	MAFB
	MAFF
	MAFG
	MAFK

Supplementary Table 3. Primer sequences used for qRT-PCR experiments

Gene	5'-Forward primer-3'	5'-Reverse primer-3'
ACTB	GCTCCTCCTGAGCGCAAG	CATCTGCTGGAAGGTGGACA
GAPDH	TGTTCGACAGTCAGCCGCATCTTC	CAGAGTTAAAAGCAGCCCTGGTGA
SOX2 endo	CGAGGGAAATGGGAGGGGTGC	TGCAGCTGTCATTTGCTGTGGGT
SOX2 viral	GCATGACCAGCAGCCAGACCTA	TCTTGACCACGCTGCCCATGCT
NANOG endo	GCCTGTGATTTGTGGGCCTGA	GTGGAAGAATCAGGGCTGTCCTG
OCT4 endo	TGTCTCCGTCACCACTCTGGGC	CCCAAAAACCCTGGCACAAACTCC
OCT4 viral	AACCCCGAGGAAAGCCAGGACA	ACAGCACGCCCAGTGTCAGT
C-MYC endo	GCGGGCACTTTGCACTGGAACT	TTTCAGAGAAGCGGGTCCTGGCA
C-MYC viral	TACGCCCTGTTGAAGCTGGCTG	TGCACCGAGTCGTAGTCGAGGT
C-MYC total	ACCGAAAATGCACCAGCCCCA	CGATCTGGTCACGCAGGGCAAA
KLF4 endo	TCCCGCCGCTCCATTACCAA	TTTTGCCGCAGCCCGCGTAA
KLF4 viral	TGGAAGTTCGCCAGAAGCGACG	TTCATGTGCAGAGCCAGGTGGT
dTOMATO	TGAAGATGCGCGGCACCAACT	TGGTGGATCTCGCCCTTCAGCA
SDHA2	GAAGCCCTTTGAGGAGCACT	GTTTTGTCGATCACGGGTCT
AIF1	AGACGTTCAGCTACCCTGACTT	GGCCTGTTGGCTTTTCCTTTTCTC
PTPRC	GCAGCTAGCAAGTGGTTTGTTC	AAACAGCATGCGTCCTTTCTC
CD68	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC
ITGAM	TGCTTCCTGTTTGGATCCAACCTA	AGAAGGCAATGTCACTATCCTCTTGA
CX3CR1	CTTACGATGGCACCCAGTGA	CAAGGCAGTCCAGGAGAGTT
TREM2	TCAGGAAGGTCCTGGTGGA	GGGTGGGAAGGGGATTTCTC
TYROPB	TACGGCCTCTGTGTGTTGAG	CGGAAACAGCGTATCACTGAG
P2RY12	TTTGTGTGTCAAGTTACCTCCG	CTGGTGGTCTTCTGGTAGCG
GPR34	CCTGATGTCCAGTAACATTCGC	CATGCAGGGAGTATCCTGGT
PROS1	TTGCACTTGTAAACCAGGTTGG	CAGGAACAGTGGTAACTTCCAG
MERTK	CTCTGGCGTAGAGCTATCACT	AGGCTGGGTTGGTGAAAACA
GAS6	CTCTCTCTGTGGCACTGGTA	CCTTGATCTCCATTAGGGCCAA
HLADRA	CCCAGGGAAGACCACCTTT	CACCCTGCAGTCGTAAACGT
TLR2	ATCCTCCAATCAGGCTTCTCT	GGACAGGTCAAGGCTTTTTACA
TLR3	CAAACACAAGCATTCGGAATCTG	AAGGAATCGTTACCAACCACATT
TLR4	AGTTGATCTACCAAGCCTTGAGT	GCTGGTTGTCCCAAAATCACTTT
TMEM119	CTTCCTGGATGGGATAGTGGAC	GCACAGACGATGAACATCAGC
GFAP	AGGTCCATGTGGAGCTTGAC	GCCATTGCCTCATACTGCGT
TUBB3	GGCCTTTGGACATCTCTTC	CTCCGTGTAGTGACCCTTG
MAP2	CTCAGCACCGCTAACAGAGG	CATTGGCGCTTCGGACAAG
OLIG2	AGGACAAGAAGCAAATGACAG	TCCATGGCGATGTTGAGG
IL6	TGCAATAACCACCCCTGACC	TGCGCAGAATGAGATGAGTTG
IL1B	TTTGAGTCTGCCCAGTTCCC	TCAGTTATATCCTGGCCGCC
CD163	TTTGTCAACTTGAGTCCCTTCAC	TCCCGCTACACTTGTTTTCAC
MRC1	TGCAGAAGCAAACCAAACCTGTAA	CAGGCCTTAAGCCAACGAAACT
TNF	TGGAGAAGGGTGACCGACTC	TCACAGGGCAATGATCCCAA
RUNX1	AAGACCCTGCCCATCGCTTT	CATCATTGCCAGCCATCACAG
SPI1	GTGCAAAATGGAAGGGTTTCCC	TACTCGTGCGTTTGGCGTTG
CSF1R	ATCAGCATCCGGCTGAAAGT	CTCGAATCCGCACCAGCTCT
IL34	TGCACTGTCACGGGTTTTCT	CCCTCGTAAGGCACACTGAT
CSF1	GCAGGAGTATCACCGAGGAG	CACGAGGTCTCCATCTGACTG
TGFB1	CAATTCCTGGCGATACCTCAG	GCACAACTCCGTGACATCAA
IRF8	ATCAAAAGGAGCCCTTCCCC	ATCAAAAGGAGCCCTTCCCC
TGFBR1	TCCAAACCACAGAGTGGGAAC	TCCAAACCACAGAGTGGGAAC
TGFBR2	GTATCGCCAGCACGATCCCA	GAAACTTGACTGCACCGTTGTT
C1QA	GAGCACCAGACGGGAAGAAA	TAAGGCCTTGGATGCCTGTC

Supplementary Table 4. Antibodies used in this study for immune histo/cytochemistry

Antigen/target	Host species	Dilutions	Provider, article number
SMA	Mouse	1:100	Sigma, A2547
AFP	Rabbit	1:50	Quartett, 2011200530
Brachyury	Goat	1:1000	R&D systems, AF2085-SP
PAX6	Mouse	1:200	DSHB, Pax6-s
NEUN	Mouse	1:300	Abcam, AB104224
TUJ1	Rabbit	1:1000	Sigma, T2200
TUJ1	Mouse	1:1000	Covance, MMS-435P
IBA-1	Rabbit	1:1000	Wakko, 019-19741
IBA-1	Goat	1:1000	Abcam, AB5076
FOXG1	Rabbit	1:100	Abcam, AB18259
CD68	Rabbit	1:100	Invitrogen, MA5-13324
CTIP2	Rat	1:100	Abcam, AB18465
TBR1	Rabbit	1:100	Gift from Robert Hevner
S100β	Rabbit	1:600	Dako, Z0311
GFAP-pan	Rabbit	1:1000	Dako, Z0334
PSD-95	Mouse	1:300	NeuroMab, 75028
MAP2	Mouse	1:300	Biolegend, SMI-52p
PU.1	Rabbit	1:100	Invitrogen, A13971
SATB2	Rabbit	1:300	Abcam, AB34735
NANOG	Rabbit	1:200	STEMLight iPSC characterization Kit, Cell Signalling, 9656S
OCT4	Rabbit	1:200	
SSEA4	Mouse	1:200	
TRA1-60	Mouse	1:200	
TRA1-81	Mouse	1:200	
SOX2	Rabbit	1:200	
Mouse, 568	Donkey	1:1000	ThermoFisher, A10037
Mouse, 555	Donkey	1:1000	ThermoFisher, A31570
Mouse, 488	Donkey	1:1000	ThermoFisher, A21202
Rabbit, 568	Donkey	1:1000	ThermoFisher, A10042
Rabbit, 488	Donkey	1:1000	ThermoFisher, A21206
Goat, 488	Donkey	1:1000	ThermoFisher, A11055
Rat, 488	Donkey	1:1000	ThermoFisher, A21208
Rabbit, Atto647N	Goat	1:200	Sigma Aldrich, 40839
Rabbit, 488	Goat	1:200	ThermoFisher, A11034
Mouse, 594	Goat	1:200	ThermoFisher, A11032